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Hebrew University-Hadassah Medical School
Environmental Health Laboratory
Jerusalem (Israel)

Final Progress Report

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by

Eliyahu Katzenelson

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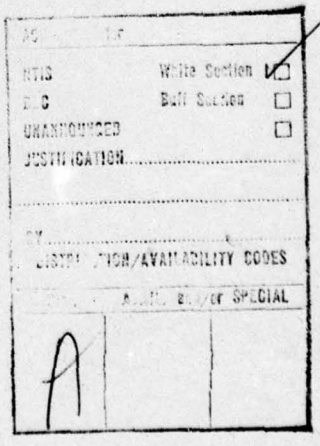

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20(U) A rapid method for the isolation and identification of viruses from water has been developed. This method allows for the demonstration of viruses in the water sample after only 6-9 hours of incubation, whereas, a time interval of 18-24 hours should satisfy most requirements for water distribution. One must take into account, however, the possibility of 'false negatives' or 'false positives' in the high speed determinations, probably due to impurities of the reagent used in the technique. Improving the quality of the reagents should permit reliability in these fast results.

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Abstract

A method is described for the rapid quantitative demonstration of polioviruses in water with the aid of the fluorescent antibody technique. Identification of the virus is possible after 18-24 hours as compared to 3-5 days required with the plaque count method. Approximately 10 plaque forming units, concentrated from a volume of 40 liters of seeded tap water could be demonstrated by the rapid method. Positive cells were already seen after 6-9 hours; the results were, however, not sufficiently quantitative. The method also showed itself to be less susceptible to bacterial contamination than the current isolation methods. Its possible utilization as a rapid, primary test for viral contamination of potable water is discussed.

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A RAPID METHOD FOR THE QUANTITATIVE ISOLATION OF VIRUSES FROM POLLUTED WATER

INTRODUCTION

The accepted techniques for virus isolation involve inoculation of tissue cultures followed by incubation. Virus is then demonstrated by the appearance of cytopathic effect of plaques in the tissue cultures. The entire process continues for 3-7 days or sometimes even longer, depending on the method.

It is obvious that a method for testing potable water sources requiring, at least, 3 days does not provide an adequate degree of protection, especially when contaminated river water is distributed to large populations within hours after passing through a treatment plant. Such tests would be as rapid as possible, and the assay should be completed before the water is released to the distributing system.

Rapid qualitative methods for demonstration of enteroviruses based on the fluorescent antibody (FA) technique have already been described.[1,2,3,4] In this study efforts were made to develop it one step further, thus affording a rapid quantitative determination of enteric viruses in water. As a model, poliovirus type I was chosen.

MATERIALS AND METHODS

Virus Stock

Poliovirus type I (Brunhilde) was used throughout this study. The virus was grown in Vero cells (Flow Laboratories, Scotland), accumulated and concentrated by the phase separation method.[5,6] Vials containing 0.5 ml of the concentrated viruses were stored at -80°C. Before each test a sample was thawed and diluted according to the experimental requirements. The virus was assayed on BGM cells,[7] as described elsewhere.[8]

Antiserum

Rabbits were injected in the footpad with 1.0 ml emulsion containing equal volumes of concentrated poliovirus I (2.3×10^{10} pfu/ml) and complete Freund's adjuvant (Difco Laboratories). Second, third and fourth identical injections were

administered after 2, 6 and 10 weeks, respectively. Fourteen days after the last injection, blood was withdrawn from the ear veins and the serum separated.

Purification and Concentration of Gammaglobulin

This was carried out as described elsewhere.[9]

Labeling of Gammaglobulin

Labeling was performed as described previously,[10] with the exception of the temperatures (about 20°C) and the stirring which was continued for 4 hrs. Isomer I of fluoresceinisothiocyanate (FITC: BDH, England) was used throughout this study.

Vero Cell Powder

Powder was prepared by acetone-drying from saline-suspended cells in the same manner as with liver powder.[11]

Removal of Non-specific Staining

The labeled gamma globulins were adsorbed by the Vero cell powder to remove non-specific staining in the following manner: 100 mg powder were suspended in saline and centrifuged at 1000 x g for 15 min; 5 ml labeled gamma globulin were added to the sediment and mixed. This suspension was incubated at 37°C for one hour, followed by centrifugation at 40,000 x g for 30 min. The supernatant was considered adsorbed serum. Additional adsorptions were sometimes necessary until complete removal of non-specific staining was achieved. A solution of rhodamine-labeled bovine albumin (Microbiological Associates) was added to the adsorbed serum (final dilution 1:40) to obtain a clear differentiation between positive and negative reactions.

Micro Tissue Culture for FA Staining

A suspension was prepared containing 2-3 x 10^6/ml BGM cells in M-199 medium with Hank's salts (Flow Laboratories, Scotland), 20% fetal bovine serum and antibiotic solution (penicillin 200 units, streptomycin 200 µg, kanamycin 5 µg, neomycin 4 µg per milliliter final concentration). This mixture was inoculated with virus according to the requirements of each experiment. Five drops (0.02 ml/drop) were put on a standard microscope slide, several slides per experiment. The slides were then placed into specially designed vessels containing small volumes of water to prevent dehydration, and incubated in a CO_2 incubator at 37°C.

Unless otherwise stated, the length of incubation was 18-20 hrs. After incubation, the slides were rinsed with 0.15 M phosphate buffer, pH 7.2, followed by three washings in acetone.

Fluorescent Antibody Staining

The direct method was used,[11] with the staining continuing for one hour. The stained preparations were examined under a Zeiss WL Research microscope with fluorescent attachment.

Concentration of Viruses from Water and Their Inoculation into Tissue Cultures

Viruses were concentrated from the water by filtration through a cellulose nitrate membrane filter (pore size 0.45 µ; Sartorius Co., Germany). For volumes of 5 liters, the technique of Rao and Labzoffsky[12] was utilized. The membrane diameter was 47 mm. The adsorbed virus was eluted form the membrane with 7 ml 3% beef extract.[13] For the testing of 40L volumes, concentration of the viruses was carried out in two stages, according to Sobsy et al,[14] with the following modifications: the pH of the water sample was adjusted to 3.0 and filtered through a cellulose nitrate membrane filter (diameter 142 mm, pore size 0.45µ). To elute the adsorbed virus, 100 ml glycine buffer (0.2 M, pH 11.5) was filtered through the membrane. The pH of the eluate was adjusted to 3.0 with 1 M HCl, and the suspension was again filtered (filter diameter 47 mm). The adsorbed virus was eluted with 7 ml 3% beef extract.[13]

One ml sterile M-199 medium (concentrated 10 fold), 2 ml fetal bovine serum and 0.1 ml antibiotic mixture (200,000 U penicillin, 200,000 µg streptomycin, 5000 µg kanamycin, 4000 µg neomycin per ml) were added to each of the 7 ml concentrate obtained in both procedures. BGM cell (0.1 ml, 4-6 x 10^6) were added to 2 ml of the concentrate.

Micro tissue cultures were prepared on microscope slides with this virus/cell suspension as described above. The remainder of the virus was used for inoculation of the tissue cultures on plates.[8]

RESULTS

Growth of Poliovirus in Micro Tissue Cultures

To determine the optimal time required for FA staining of the cultures, infected micro tissue cultures were prepared and incubated. The first slide was removed

after incubation of one hour, washed, fixed and stained. The second slide was treated in the same manner after incubation of two hours. This procedure was followed for 24 consecutive hours. All slides were examined microscopically. Incubation of one hour showed the cells to be attached to the slide; nearly all were still spherical and separated one from the other. All cells stained reddish-brown, the color of the rhodamine bovine albumine used as the counter stain.

Four hours after incubation, the cells appeared as in mature tissue cultures; they were flattened and attached to one another, giving the impression of monolayers. The cells were still stained with the counter stain.

Individual cells stained with the green fluorescence of the fluorescent antibodies started to appear after 6-7 hours of incubation, and after 9 hours a miximum of individual stained cells was reached. They appeared as isolated green spheres, surrounded by the reddish brown stained cells (Fig 1).

After an incubation period of 16 hours the positive cells started to appear in foci of 5-30 cells, looking like clusters of green fluorescent spheres (Fig 2). The number of positive cells increased in each infected foci until, after 22-24 hours, the clusters had grown into small plaques, each comprising scores of cells.

Quantitative Estimation of Poliovirus with FA Staining

The foregoing experiments indicated that the growth process of poliovirus in micro tissue cultures, as revealed by the FA staining, can be divided into two stages: stage 1, reaching its peak at 9 hours, during which positive cells remain single entities, and stage 2, with the peak at about 18-24 hours, when the positive cells form clusters. It is reasonable to assume that both the single positive cells appearing at 9 hours and the clusters at 18-24 hours, each represent one plaque forming unit of the original suspension. To test this contention, a number of experiments were carried out. Micro tissue cultures, infected with serial 10-fold dilutions of poliovirus, were prepared as described in methods and divided into two batches. One batch was incubated for 9 hours and the other for 20 hours. The FA stained preparations were examined throughly under the microscope. The single positive cells (9 hrs) and the clusters of positive cells (20 hrs) were counted. Simultaneously, the same poliovirus stock was titrated on plates as a control (Table 1). There is agreement between

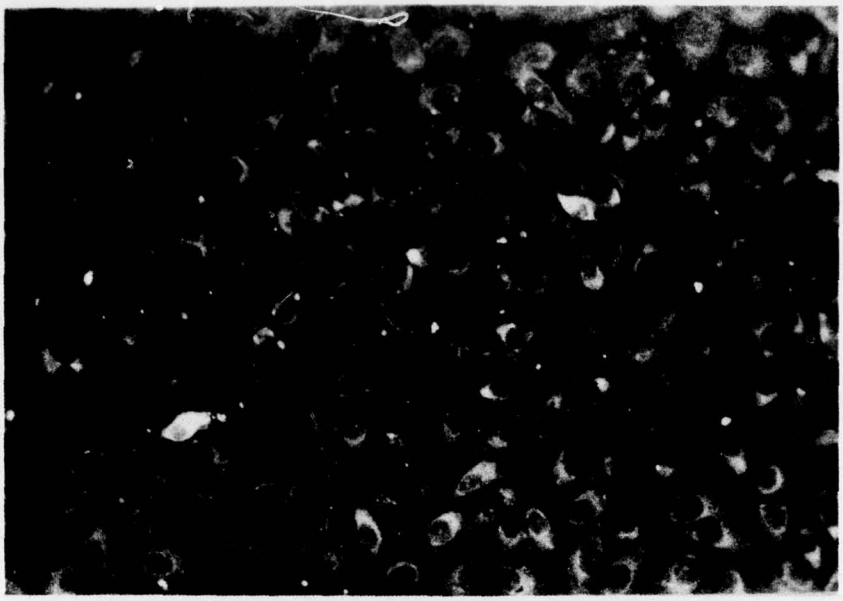

Fig 1 BGM cells infected with poliovirus type I, stained with fluorescent antibodies; 9 hours after infection. Two positive (white) are clearly seen. (x 100).

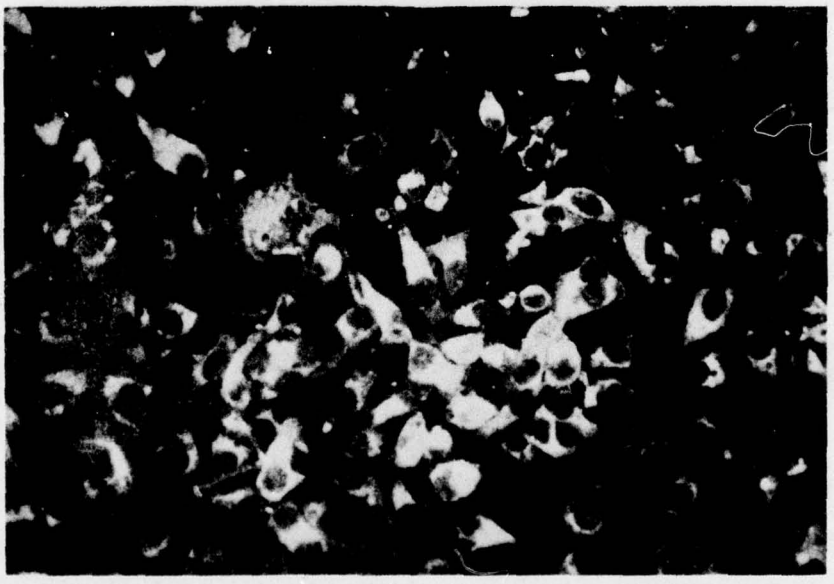

Fig 2 BGM cells infected with poliovirus type I, stained with fluorescent antibodies; 18 hours after infection. The cluster of positive cells is clearly defined. (x 100).

the FA preparations incubated for 20 hours and the controls. The 9-hour incubate, however, shows less satisfactory results, with a virus titer far below that of the controls. Another important factor with the 20-hour incubate was the readily discernible clusters, which should enable any technician with minimal experience to read results with ease. The single stained cells (9 hours), on the other hand, are often difficult to recognize or to distinguish from non-specific fluorescence. In view of these obstacles, 9-hour incubates were discontinued and the results described below concern only samples taken at 18-24 hours.

Table 2 compares the poliovirus titers of the FA stained cells and the plaque assay method. The results of the plaques were read two days after those of the FA. The virus titers in both methods are nearly identical.

Quantitative Detection of Poliovirus in Water

After it was established that the above procedure enables a rapid and quantitative assay of poliovirus in controlled experiments, it was decided to investigate its practical application--that is, the quantitative isolation of the virus from large volumes of water. Two accepted methods for the concentration of viruses from water were utilized in this study: one designed for volumes of 5 liters, and the other for larger bodies of water, in our case, a volume of 40 liters. Tap water was contaminated with 10-1000 pfu poliovirus I, according to the requirements of the experiment, concentrated and titrated simultaneously on micro cultures and plates. Tables 3 and 4 summarize the results of experiments with the 5- and 40-liter samples, giving good correlation between the two methods. Because of contamination, no results could be obtained in three experiments with the plaque assay, a factor which did not play with the FA method (Table 4).

DISCUSSION

The main reason for virus monitoring of water is to protect the population from health hazards. It would be desirable if the quality of the water be known before it reaches the consumer. Since impoundment of vast quantities of water for long periods is undesirable, rapid results of water tests are imperative.

Standard bacteriological tests require 24 hours, but even this relatively brief period should be shortened for most water distribution systems. Virological tests which take days, and sometimes weeks, are, of course, even less efficient in providing early warning of contamination. The reason for this time lag is

Table 1. Titers of poliovirus I obtained with the fluorescent antibody technique after 9 and 20 hour incubation, and with the plaque count method on plates

Exp. No.	Incubation period (hours)	Dilution of virus sample	No. of positive cells or cell groups per drop	Calculated virus titer	Virus titer on plates
1	20	10^{-4}	3, 4, 7, 3	2.1×10^7	
		10^{-5}	2, 1, 1, 2	7.5×10^7	3.4×10^7
	9	10^{-2}	169, 190, 164	8.7×10^6	
		10^{-3}	23, 21, 18	1.0×10^7	
2	20	10^{-4}	2, 3, 1, 2	1.0×10^7	
		10^{-5}	1, 1, 0, 1	3.8×10^7	3.0×10^7
	9	10^{-1}	78, 61, 67	3.4×10^5	
		10^{-2}	8, 5, 2	2.6×10^5	
3	20	10^{-4}	4, 3, 4, 2	1.6×10^7	
		10^{-5}	2, 2, 1, 0	6.3×10^7	2.6×10^7
	9	10^{-2}	58, 44, 53, 26	2.1×10^6	
		10^{-3}	6, 4, 3, 4	2.1×10^6	

Table 2. Titers of poliovirus type 1 obtained with the fluorescent antibody technique and with the plaque count method

Exp. No.	Virus titer	
	Plaque count	FA technique
1	3.0×10^7	2.6×10^7
2	2.7×10^7	6.2×10^6
3	1.5×10^7	1.1×10^7
4	2.0×10^7	1.2×10^7
5	1.1×10^7	1.2×10^7
6	2.2×10^7	1.3×10^7
7	1.8×10^7	1.5×10^7
8	1.4×10^7	1.4×10^7

Table 3. Comparison of the fluorescent antibody technique with the plaque count method for quantitative evaluation of viruses in 5 liters of water

Exp. No.	pfu recovered	
	FA counts	Plaque counts
1	800	920
2	800	624
3	110	942
4	90	140
5	100	150
6	100	167
7	100	133
8	28	37
9	55	35
10	55	66
11	24	66
12	20	61
13	26	44
14	32	61

Table 4. Comparison of the fluorescent antibody technique with the plaque count method for the quantitative evaluation of viruses in 40 liters of water

Exp. No.	pfu recovered	
	FA counts	Plaque counts
1	22	33
2	36	8
3	3	7
4	7	contaminated
5	21	contaminated
6	14	contaminated
7	42	6
8	8	13
9	8	19

not inherent in the concentration methods. Although some of these methods are slow, as for example, the phase separation method which needs 24-48 hours,[5,6] most concentration methods require 1-6 hours only. On the other hand, the time needed for the isolation of virus is far more extended. The usual techniques involve inoculation of tissue cultures followed by incubation. Viruses are then demonstrated by the appearance of the cytopathic effect (CPE) or plaques in the tissue cultures. The entire process lasts from 3 to 7 days or longer, depending on the particular method. In our laboratory, the plaque assay is used, which enables quantitative evaluation of the virus 3-5 days after sampling. It is possible to shorten the time needed for the plaque assay, but the danger of incomplete plaque development then exists, which is expressed by too low a number of plaques or by false negatives.

The main objective of the present study was the development of a quantitative method for the isolation of viruses from water which would be at least as rapid as the current bacteriological methods.

Viruses enter drinking water sources by way of domestic sewage. In the latter, various types of viruses are present[15] most of which belong to the enterovirus groups, including the following sub-groups: polio, coxsackie and echo viruses. They are mostly found in sewage and their concentration ranges from 400-2000 pfu/ℓ,[15] sometimes reaching over 10,000 pfu/ℓ. Of the entire enterovirus group, only the three polio types should always be present in domestic sewage of urban areas in developed countries. The reason is the wide-spread routine administration of live poliovirus vaccine to infants. The viruses multiply in the intestines and are excreted with the feces for several weeks after the initial administration. Their number in the feces may reach 10^7 pfu/gm.[16] The presence of other types of enteroviruses in sewage depends on the degree of their distribution amongst the population at a given time, and this of course fluctuates considerably. This fact should be borne in mind when water is tested for the presence of viruses. Moreover, a single system that could be used to isolate all the different types of enteroviruses does not exist. Most of the coxsackie A virus types do not multiply in tissue cultures but require suckling mice. Polio- and echovirus, on the other hand, grow in tissue cultures. Also, the length of time required for the development of the cytopathic effect varies for each virus type. For example, CPE of poliovirus is displayed in 3-5 days, while that of reoviruses appears considerably later. The isolation of the different virus types found in water requires a wide range of techniques and systems, a fact which makes routine practical use too complicated.

In bacterial examinations, only one type of bacterium--coliforms--is taken as being representative for other enteric bacteria present in the feces, thus becoming the indicator for bacterial fecal pollution. It would therefore be logical to select a viral indicator, the poliovirus being the most suitable candidate. Such an indicator for viral pollution of water could simplify the technique since it is based on a single system. Furthermore, it would also significantly shorten the identification period.

However, there are certain limitations regarding the use of poliovirus as viral indicator. Unlike E. coli, they do not necessarily comprise the majority of viral population in sewage, and at times are not present at all. Therefore, negative results obtained with tests based on polioviruses only, would not be sufficient proof for the absence of other dangerous viruses in water. On the other hand, the lack of a rapid virus test slows down the implementation of routine viral examination of potable water. The use of three polioviruses as indicators is suggested for a rapid examination of water as part of a complete and comprehensive virological test that will include all possible viruses in water. Thus, a rapid and preliminary answer may be obtained as to the presence of viruses in water designated for human consumption. With this objective in mind, polioviruses were selected for the present study, with poliovirus I as model.

The process of the development of the CPE is slow and includes the following stages: infection of susceptible cells, multiplication and liberation of the progeny, upon which the cycle starts again. In the case of polioviruses, each cycle lasts for 6-9 hrs[17] at 37°C. Several such cycles are needed before the CPE can be detected visually; in other words, the results are detectable only after a few days. A reagent that would enable identification of the virus in the tissue culture, before the CPE becomes visible, would therefore allow for a much shortened test time. Fluorescent antibodies could here fulfill the role of such a reagent.

Final identification of viruses is currently carried out either by the use of specific antibodies which neutralize the appearance of CPE in infected tissue cultures or by fluorescent antibodies. The latter stain the cells containing viral antigens, thus enabling identification of viruses in the cell considerably before the visible CPE. It is noteworthy that poliovirus antigens were already demonstrated approximately 6 hours past infection and reached their maximal concentration 3 hours thereafter. Thus, by using fluorescent antibodies it is

theoretically possible to determine the presence or absence of poliovirus in tissue cultures 6-9 hours after infection. The FA technique was suggested as a method for typing polioviruses in 1959[5] and it was later established as a reliable technique.[1,2,4] The value of this technique for the identification of poliovirus is beyond dispute.

The FA method requires a special microscope, not suitable for tissue cultures grown in bottles or on plates. A method of micro tissue cultures on microscope slides was therefore developed. The cells were inoculated with the virus while still in suspension and measured drops (0.02 ml) were placed on a slide thus making quantitative determination of the virus possible.

BGM cells,[7] used routinely in our laboratory for virus isolation from water, were employed throughout this study. It is reasonable that other types of cells may be suitable here, although the number of cells necessary to obtain a monolayer within a short period of time should then first be determined. The number of 2-3 x 10^6 cells/ml was chosen with BGM cells after trial and error. This number may not apply, however, to other cell types. Using BGM cells in the micro tissue culture method resulted in a monolayer after 4 hours. Twenty-four hours after the start of the experiment (the time needed for completion of the test) the cells still formed a monolayer.

Positive cells, stained by the FA technique, were already observed after incubation of 9 hours, but the results were not yet sufficiently quantitative. Furthermore, the finding and identifying of positive cells at a time when they are still scarce, enhanced the danger of 'false positive' or 'false negative' identification. On the other hand, after incubation of 18-24 hrs, the cell culture was at the end of the second virus growth cycle e.g., the progeny of each primarily infected cell had, in turn, infected the neighboring cells. Viewing these FA stained preparations, under the microscope demonstrated groups of positively stained cells. It is extremely simple to identify such cell groups, and even a relatively inexperienced person could easily recognize them. The results after 18-24 hrs incubation are almost identical to those obtained by the plaque count technique, with the difference that the latter method requires two additional days.

The rapid method could be utilized for various virological procedures and is not necessarily limited to water testing. Since our motivation was the virological examination of water, the proposed method was tried in combination with various techniques for concentration of viruses from water. Two accepted techniques,

based on membrane filtration[12,14] were chosen for this purpose. They are simple to perform and have a high concentration factor of 1000-10,000.

A 40-liter volume was concentrated to 7 ml of fluid. But even this small volume required scores of slides with the micro tissue culture system. Ten slides resulted in the demonstration of approximately 10 pfu/40 liters, which was the upper limit of sensitivity. Techniques with a higher concentration factor would enhance the sensitivity of the rapid method and simplify the procedure. For practical routine application, 0.5 ml final concentrate would be most suitable.

The rapid method and the accepted plaque technique, using concentrated water samples, yielded nearly identical numbers of viruses. Furthermore, apart from the fact that the rapid method showed results 2-3 days before the plaque technique. plates with the latter were often contaminated with bacteria, thus prohibiting reading of results. The incubation period in the rapid method was too short to allow contamination.

The objective of the study was to develop a rapid method for the isolation and identification of viruses from water. A time interval of 18-24 hours should satisfy most requirements for water distribution. It should be pointed out that, in fact, this rapid method allows for the demonstration of viruses in the water sample after only 6-9 hours of incubation. However, the possibility of 'false negatives' or 'false positives' has then to be taken into account, probably due to the impurities in the reagents used in the FA technique. Improving the quality of the reagents should overcome these obstacles and may enable virus identification in water within 9 hours or less.

REFERENCES

1. Brown, G.C. Fluorescent Antibody Techniques for the Diagnosis of Enteric Infections. Arch. Ges. Virusforsch. 13:30-34, 1963.
2. Hatch, M.H., S.S Kalter and G.W. Ajello. Identification of Poliovirus Isolates with Fluorescent Antibody. Proc. Soc. Exp. Biol. Med. 107:1-4, 1961.
3. Kalter, S.S., M.H. Hatch, and G.W. Ajello. The Laboratory Diagnosis of Polimyelitis with Fluorescent Antibodies. Bacteriol. Proc. p. 89-90, 1959.
4. Riggs, J.L., and G.C. Brown. Application of Direct and Indirect Immunofluorescnece for Identification of Enteroviruses and Titrating Their Antibodies. Proc. Soc. Exp. Biol. Med. 110:833-837, 1962.
5. Shuval, H.I., S. Cymbalista, B. Fattal and N. Goldblum. Concentration of Enteric Viruses in Water by Hydro Extraction and Two Phase Separation. In: Transmission of Viruses by the Water Route, Berg, G., (ed.). New York, Interscience Publ., 1967. p. 45-55.
6. Shuval, H.K., B. Fattal, S. Cymbalista, and N. Goldblum. The Phase Separation Method for the Concentration and Detection of Viruses in Water. Water Res. 3:225-240, 1969.
7. Barron, A.L., C. Olshevsky, and M.M. Cohen. Characteristic of the BGM Line of Cells from African Green Monkey Kidney. Arch. Ges. Virusforsch. 32:389-392, 1970.
8. Shuval, H.I., A. Thompson, B. Fattal, S. Cymbalista, and Y. Wiener. Natural Virus Inactivation Processes in Seawater. J. San. Eng. Div. ASCE 97:587-600, 1971.
9. Katzenelson, E., and H. Bernkopf. Studies of the P.L.T. Agents with the Aid of the Agar Immunodiffusion Technique. Amer. J. Opthal. 63:1483-1487, 1967.
10. Katzenelson, E., and H. Bernkopf. Serologic Differentiation of Trachoma Strains and other Agents of the P.L.T. Group with the Aid of the Direct Fluorescent Antibody Method. J. Immunol. 94:467-474, 1965.
11. Narin, R.C. Fluorescent Protein Tracing. 3rd Ed. London, E. and S. Livingstone, Ltd., 1969.
12. Rao, N.U., and N.A. Labzoffsky. A Simple Method for the Detection of Low Concentrations of Viruses in Large Volumes of Water by the Membrane Filter Technique. Can. J. Microbiol. 15:399-403, 1969.
13. Berg, G., D.R. Dahling, and D. Berman. Recovery of Small Quantities of Viruses from Clean Waters on Cellulose Nitrate Membrane Filters. Appl. Microbiol., 22:608-614, 1971.

14. Sobsey, M.D., C. Wallis, M. Henderson, and J.L. Melnick. Concentration of Enteroviruses from Large Volumes of Water. Appl. Microbiol. 26:529-534, 1973.

15. Shuval, H.I. Detection and Control of Enteroviruses in the Water Environment Development. In: Water Quality Research. Ann Arbor-London, Ann Arbor Humphrey Science Publ., 1970. p. 47-71.

16. Sabin, A.B. Recent Studies and Field Tests with a Live Attenuated Polio Virus Vaccine. First International Conf. on Live Polio Viruses Vaccines. Pan American Sanitary Bureau, 1959. p. 14-33.

17. Lwoff, A., R. Dulbecco, M. Vogt, and M. Lwoff. Kinetics of the Release of Poliomyelitis Virus from Single Cells. Virology. 1:128-139, 1955.